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| <b>(54) Title:</b> ENZYME STABILISATION<br><br><b>(57) Abstract</b><br><br>A method of stabilising enzymes or other proteins against denaturation or drying comprises mixing the protein with a cyclic polyol and a cationic polyelectrolyte.   |           |   |

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ENZYME STABILISATION

This invention relates to stabilisation of proteins, particularly but not exclusively of enzymes in the dry state.

Few enzymes are inherently stable in solution. Many have a tendency to become denatured when held in solution. Various workers have attempted to stabilise enzymes either by adding compounds such as sugars or glycerol to solutions of them or by freeze drying. These methods often cause a loss of activity. Alternative methods of stabilisation have involved drying of enzymes with stabilisers in a presence of a solid support such as cellulose fibre or polyacrylamide. US 4451569 disclosed stabilisation of glutathione peroxidase by freezing the enzyme with one of a number of sugars including arabinose, glucose, xylitol and sorbitol. Freeze drying is expensive to operate on a large scale and often results in denaturation.

PCT/GB86/00396 discloses stabilisation of proteins by use of the disaccharide trehalose.

According to a first aspect of the present invention a method of protecting proteins against denaturation on drying comprises mixing an aqueous solution of the protein with a soluble cationic polyelectrolyte and a cyclic polyol, and removing water from the solution.

Stabilisation in accordance with this invention enhances the activity of freshly dried enzymes and other proteins. The stability upon storage is also enhanced.

The proteins may include enzymes, antibodies, antigens, serum complement, vaccine components and bioactive peptides.

Drying of proteins and especially enzymes is important for many applications, for example use in diagnostic or analytical aids such as test strips which may be stored for prolonged periods before use. Transportation of enzymes or other proteins in solution is inconvenient and expensive.

Although freeze drying may be employed, the present invention facilitates use of the vacuum drying and air drying

without denaturation. Vacuum drying and air drying milder processes and are much cheaper to operate.

The cyclic polyol may incorporate one or more alicyclic rings and may have at least one side chain. Compounds having 5 to 10 hydroxyl groups may be preferred. Non-reducing polyols are preferred. Di and trisaccharides are particularly efficacious but other cyclic polyols, for example inositol may also be used. The polyol may be chosen to suit both the enzyme or other protein and also the polyelectrolyte in question. Lactitol, lactose, maltose and sucrose are especially preferred in conjunction with DEAE-dextran, lactitol having been found to be most suitable for many applications. Sorbitol is suitable for use with cholesterol oxidase, cholesterol esterase and other enzymes. Cellobiose may also be used. The amount of polyol may lie in the preferred range of 1 to 20%, more preferably 2 to 10%, most preferably 5 to 10%.

The cationic polyelectrolyte is preferably a polymer with cationic groups distributed along the molecular chain. The cationic groups, which are preferably quaternary ammonium derived functions, may be disposed in side groups pendent from the chain or may be incorporated in it. Natural or artificial polymers may be employed. Natural polymers such as polysaccharides are preferred since many artificial polymers contain residual traces of the inorganic polymerisation catalyst.

Diethylaminoethyl dextran (DEAE-dextran) and chitosan are preferred although polyethyleneimine is also suitable. Polysaccharides with MW 5000 to 500 000, preferably 5000 to 20 000, more preferably 5000 to 10 000 may be employed. An amount of 0.1 to 10% is preferred, especially 0.5 to 2%.

The pH at which enzymes are dried in accordance with this invention may be important to optimise retention of activity both upon drying and after subsequent storage. The optimum pH for a particular enzyme may be determined by simple experimentation.

Alcohol oxidase has been found to retain activity between pH 7 and 8, preferably at pH 7.8.

Cholesterol oxidase, dependent on the source, dries best at

pH 5 or 9.

Uricase may be dried at pH 9.

Cholesterol esterase dependent on source may be dried at pH 7 or 9.

Drying is preferably performed in the presence of a wetting agent. Temperatures between 4° and 50°, especially 25° to 35° are preferred.

According to a second aspect of the present invention there is provided a dried product containing a protein, cyclic polyol and cationic polyelectrolyte.

The dried product may be a free running powder or may comprise part of a test strip or other analytical or diagnostic apparatus.

The present invention is now described by means of example but not in any limitative sense.

#### Experimental Procedures

Percentages used in the specification are by weight unless indicated otherwise.

All the stabilisation systems utilise buffers to maintain stable pH conditions eg.

Buffer solutions containing  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (10.855g) and  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (6.084g) were dissolved in 1.0 litre distilled water to give a solution of pH 7.0 at a concentration of 100 millimoles per litre.

An alternative buffer is MOPS - (4-Morpholine Propane Sulphuric Acid) - 52.25g/2.5l distilled water pH to 7.87 with 4.0M.NaOH.

A wetting agent may be used dependent on whether or not the enzyme system is being stabilised in a polystyrene cuvette. A suitable wetting agent is protein hydrolysate from gelatine termed Byco A. These are made up to 1% w/v in phosphate buffer, 100  $\text{mmol.l}^{-1}$ , pH 7.0 as needed.

Enzyme solutions were made up freshly before use. Stock solutions of enzymes in ammonium sulphate solution were dialysed

exhaustively against buffer eg  $100 \text{ mmol.l}^{-1}$  phosphate buffer pH 7.0 to remove all salts.

Stock enzyme concentrations may be from 10 to 1000 units of activity per millilitre of solution. In terms of protein concentration this is between  $0.5$  to  $200 \text{ mgcm}^{-3}$ . Typically the final protein concentration was  $1.0 \text{ mgcm}^{-3}$ .

Soluble polyelectrolytes, polyols, enzyme, buffer salts and wetting agent (if used) were mixed at constant temperature and dried in a vacuum oven over dessicant eg. silica gel,  $0.1 \text{ mm/Hg}$ ,  $30^\circ\text{C}$  for 4-10 hr.

The oxidase enzymes studied may be assayed by colorimetric detection of the hydrogen peroxide produced by action of the enzyme. Peroxidase acts on the hydrogen peroxide produced in the presence of aromatic alcohols or amines and the heterocyclic compound 4-aminoantipyrine to give quinoneimine dyes. Other standard assays systems may be employed eg u.v. spectrometry.

The following systems were employed:

#### System 1

|                       |                           |
|-----------------------|---------------------------|
| Phenol sulphonic acid | $25 \text{ mmol.l}^{-1}$  |
| 4-aminoantipyrine     | $0.4 \text{ mmol.l}^{-1}$ |
| Peroxidase            | 1000 unit/l               |

The resultant dye was measured at 500nm.

#### System 2

|   |                           |
|---|---------------------------|
| 3,5-dichloro 2-hydroxybenzene<br>sulphonic acid | $10 \text{ mmol.l}^{-1}$  |
| 4-aminoantipyrine                               | $0.4 \text{ mmol.l}^{-1}$ |
| Peroxidase                                      | 1000 units/l              |

The resultant dye was measured at 520 nm.

Standard temperature eg.  $25^\circ\text{C}$  and incubation times eg. 5

minutes were used. Reagent blanks contained all components except substrate. Dry preparations in cuvettes were reconstituted with Systems 1. or 2. directly.

Dry powdered preparations were reconstituted with phosphate buffer and suitable aliquots were added to System 1. or 2.

For stability trials the storage temperature was 37°C, with samples being removed periodically to check for residual activity of the enzyme. This procedure was standard for all enzymes tested.

#### Soluble Polyelectrolyte and Sugar Alcohol or Saccharide

##### Soluble Polyelectrolyte

Soluble polyelectrolyte was dissolved in distilled water a concentration up to 20% w/v, usually to 10% w/v. Sugar alcohol or saccharide was dissolved in distilled water up to a concentration of 40% w/v, usually to 20% w/v. These solutions were used within 4 weeks of preparation, being stored in the cold at 4°C.

##### Example 1

|            |  |       |
|------------|--|-------|
| Solution 1 | DEAE-Dextran 10%                         | 100ul |
|            | Lactitol 20%                             | 500ul |
|            | Byco A 1%                                | 100ul |
| Solution 2 | Alcohol oxidase 7 units                  | 35ul  |
|            | (1.7mg protein                           |       |
|            | Phosphate buffer 100mmol.l <sup>-1</sup> | 265ul |
|            | pH 7.0                                   |       |

Solution 1 was stirred continuously whilst slowly adding Solution 2 at 4°C. The mixture was stirred for 5 minutes to ensure complete mixing. 0.1ml volumes were dried in cuvettes as described, stored at 37°C and assayed for activity as described

(Table 1).

Example 2

|            |   |   |
|------------|---|---|
| Solution 1 | Alcohol oxidase 2 411 units<br>(=422 mg protein) in phosphate buffer<br>300mmol.l <sup>-1</sup> | 2.7cm <sup>3</sup>                        |
| Solution 2 | Lactitol 20% w/v<br>DEAE-Dextran 10% w/v  | 3.0cm <sup>3</sup><br>0.27cm <sup>3</sup> |

Solution 2 was added slowly to Solution 1 with stirring. The mixed solutions were pipetted into petri dishes and vacuum dried over silica gel at 30°C for 8 hours whereupon a thin glassy film of dried enzyme and stabiliser was produced. This was removed and ground to a fine powder using a glass pestle and mortar.

For stability testing 10mg portions of enzyme powder were weighed into sterile polystyrene tubes and incubated at 37° in a sealed container over silica gel. Samples were removed periodically and reconstituted in distilled water. 60ul of reconstituted enzyme solution was added to each assay cuvette containing peroxidase and colour reagents as described (Table 2).

Example 3

|            |  |                         |
|------------|--|-------------------------|
| Solution 1 | DEAE-Dextran 10% w/v<br>Lactitol 20% w/v<br>Byco A 1% w/v                                | 100ul<br>500ul<br>100ul |
| Solution 2 | Choline oxidase 10 units<br>(0.794 mg protein)<br>in phosphate pH 7.0 100cm <sup>3</sup> | 300ul                   |

Solution 2 was added with stirring to Solution 1 and thoroughly mixed at 4°C. 0.1cm<sup>3</sup> volumes were vacuum dried in



cuvettes as described, stored at 37°C and assayed for activity as described (Table 3).

#### Example 4

##### Glycerol 3 Phosphate Oxidase

|            |  |       |
|------------|--|-------|
| Solution 1 | DEAE-Dextran 10% w/v                               | 100ul |
|            | Lactitol 20% w/v                                   | 500ul |
|            | Byco A 1% w/v                                      | 100ul |
| Solution 2 | Glycerol 3 Phosphate Oxidase                       |       |
|            | 10 units (0.526 mg protein)                        | 300ul |
|            | in phosphate buffer pH 7.0 100mmol.l <sup>-1</sup> |       |

Solution 2 was added with stirring to Solution 1 at 4°C and thoroughly mixed. 0.1cm<sup>3</sup> volumes were vacuum dried in cuvettes as described (Table 4).

#### Example 5

|            |   |       |
|------------|---|-------|
| Solution 1 | DEAE-Dextran 10%                          | 100ul |
|            | Lactose 20%                               | 500ul |
|            | Byco A 1%                                 | 100ul |
| Solution 2 | Alcohol oxidase 5 units                   | 300ul |
|            | (1.0mg protein in 100mmol.l <sup>-1</sup> |       |
|            | Phosphate buffer pH 7.0                   |       |

Solution 1 was stirred continuously whilst slowly adding Solution 2 at 4°C. The mixture being stirred for 5 minutes to ensure complete mixing 0.1cm<sup>3</sup> volumes were dried in cuvettes as described (Table 5).

#### Soluble Polysaccharides

Soluble polysaccharides were dissolved in distilled water up to a concentration of 30% w/v usually to a concentration of 10% w/v. These solutions were used within 4 weeks of preparation and stored at 4°C.

#### Example 6

|            |  |       |
|------------|--|-------|
| Solution 1 | Dextran (molecular wt. 10 000)               | 100ul |
|            | 10% w/v                                      |       |
|            | Byco A 1% w/v                                | 100ul |
|            | Distilled water                              | 500ul |
| Solution 2 | Alcohol oxidase 7 units                      |       |
|            | (1.32 mg protein) in 100mmol.l <sup>-1</sup> | 300ul |
|            | Phosphate buffer pH 7.0                      |       |

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing. 0.1cm<sup>3</sup> volumes were vacuum dried in cuvettes, stored at 37°C and assayed for activity as described.

When dextrans of differing molecular weights are used variations in stability were noted (Table 6).

#### Example 7

|            |  |       |
|------------|--|-------|
| Solution 1 | Dextran molecular wt. 10 000               | 500ul |
|            | 10% w/v solution                           |       |
|            | Phosphate buffer 10mmol.l <sup>-1</sup>    | 300ul |
|            | pH 7.0                                     |       |
| Solution 2 | Galactose oxidase 0.52 units               |       |
|            | (0.8 mg protein) in 10mmol.l <sup>-1</sup> | 200ul |
|            | phosphate buffer pH 7.0                    |       |

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing.

0.1 ml aliquots were vacuum dried, stored at 37°C and assayed for activity as described (Table 7).

#### Cyclic Polyalcohol

Cyclic polyalcohol was dissolved in distilled water to a concentration of 10% w/v. The solutions were stored at 4°C and used within 4 weeks of preparation.

#### Example 8

|            |                                |       |
|------------|--------------------------------|-------|
| Solution 1 | Inositol 10% w/v               | 500ul |
|            | Distilled water                | 200ul |
| Solution 2 | Alcohol oxidase 4.7 units      |       |
|            | 91.15mg/protein) in Phosphate  | 200ul |
|            | buffer 100mmol.l <sup>-1</sup> |       |

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing. 0.1cm<sup>3</sup> aliquots were vacuum dried, stored at 37°C and assayed for activity as described (Table 8).

#### Example 9

|            |   |       |
|------------|---|-------|
| Solution 1 | Inositol 10% w/v                          | 500ul |
|            | Phosphate buffer 100mmol.l <sup>-1</sup>  |       |
|            | pH 7.0                                    | 300ul |
| Solution 2 | Galactose oxidase 0.52 units              |       |
|            | (0.8mg protein) in 10mmol.l <sup>-1</sup> |       |
|            | phosphate buffer pH 7.0                   | 200ul |

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing. 0.1cm<sup>3</sup> aliquots were vacuum dried as described, stored at 37°C

and assayed for activity as described (Table 9).

Example 10

The following results show the stabilisation of alcohol oxidase (*Hansenula polymorpha*).

Unstabilised enzyme retained 26% activity after 7 days incubation at 37°C. Addition of chitosan above gave retention of 48.9% activity after 9 days. The activity in relation to freshly dried enzyme was measured after incubation at 37°C.

| Stabiliser             | Period of incubation<br>/days | Activity/% |
|------------------------|-------------------------------|------------|
| lactitol 5%            | 1                             | 86.9       |
| chitosan 0.1%          | 6                             | 85.7       |
|                        | 9                             | 82.1       |
|                        | 16                            | 86.1       |
| lactitol 5%            | 1                             | 87.4       |
| chitosan 0.01%         | 6                             | 87.2       |
|                        | 9                             | 83.4       |
|                        | 16                            | 91.6       |
| lactitol 5%            | 1                             | 79.3       |
| polyethyleneimine 0.1% | 6                             | 77.5       |
|                        | 9                             | 76.1       |
|                        | 16                            | 77.5       |

## 11

|                         |    |      |
|-------------------------|----|------|
| lactitol 5%             | 1  | 91.1 |
| Polyethyleneimine 0.01% | 6  | 84.4 |
|                         | 9  | 96.1 |
|                         | 16 | 93.1 |

---

|                   |    |      |
|-------------------|----|------|
| lactitol 5%       | 1  | 94.9 |
| DEAE-Dextran 0.1% | 6  | 85.1 |
|                   | 9  | 88.7 |
|                   | 16 | 90.6 |

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|                    |    |      |
|--------------------|----|------|
| lactitol 5%        | 1  | 98.3 |
| DEAE-Dextran 0.01% | 6  | 88.8 |
|                    | 9  | 89.4 |
|                    | 16 | 95.9 |

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Example 11

The following results show stabilisation of alcohol oxidase (*Pichia pastoris*). Unstabilised enzyme retained 49.8% and 36.1% activities after 2 days and 13 days respectively at 37°C. Enhanced activity (ie greater than 1-%) upon drying may be attributable to selective degradation of inhibiting impurities.

---

| Stabiliser      | Period of incubation<br>days | Activity/% |
|-----------------|------------------------------|------------|
| lactitol 5%     | 1                            | 102.5      |
| DEAE-Dextran 1% | 4                            | 116.6      |

---

|             |    |       |
|-------------|----|-------|
|             | 8  | 121.3 |
|             | 15 | 104.3 |
| <hr/>       |    |       |
| dextran 5%  | 1  | 83.2  |
|             | 4  | 97.0  |
|             | 8  | 101.7 |
|             | 15 | 87.6  |
| <hr/>       |    |       |
| inositol 5% | 1  | 88.0  |
|             | 4  | 106.2 |
|             | 8  | 107.1 |
|             | 15 | 109.1 |
| <hr/>       |    |       |

Example 12

The following results illustrate stabilisation of cholesterol oxidase (*Nocardia erythropolis*). Unstabilised enzyme retained 34.3% activity after 14 days at 37°C.

| Stabiliser      | Period of incubation<br>days | Activity/% |
|-----------------|------------------------------|------------|
| <hr/>           |                              |            |
| lactitol 5%     | 3                            | 96.2       |
| DEAE-dextran 1% | 5                            | 105.6      |
|                 | 14                           | 115.7      |
| <hr/>           |                              |            |
| inositol 5%     | 1                            | 92.6       |
|                 | 7                            | 84.8       |
|                 | 10                           | 91.7       |

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Example 13

The following results illustrate stabilisation of freeze dried uricase.

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| Stabiliser      | Period of incubation<br>days | Activity/% |
|-----------------|------------------------------|------------|
| <hr/>           |                              |            |
| lactitol 5%     | 1                            | 109.9      |
| DEAE-dextran 1% | 5                            | 114.3      |
|                 | 10                           | 109.9      |

---

Example 14

The following results illustrate stabilisation of various enzymes with lactitol (15%) and DEAE-dextran (1%) during drying in comparison to the activity of undried enzymes.

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| Enzyme | Activity after drying/% |            |
|--------|-------------------------|------------|
|        | Unstabilised            | Stabilised |

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|  |      |                       |
|--|------|-----------------------|
| Alcohol oxidase<br>(Pichia)                  | 64.7 | 78.2                  |
| Choline oxidase                              | 63.3 | 97.7                  |
| Lactate oxidase                              | 77.1 | 90.0                  |
| Alcohol oxidase<br>(Hansenula<br>polymorpha) | 68.2 | 119.6                 |
| Cholesterol<br>oxidase<br>(vacuum dried)     | 80.0 | 92.5<br>(inositol 5%) |
| (freeze dried)                               | 79.0 | 91.0<br>(inositol 5%) |

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TABLE 1

| Preparation  | Incubation<br>37°C | % Activity remaining<br>relative to activity<br>freshly dried enzyme |
|--|--------------------|--|
| Alcohol oxidase +<br>Lactitol 10%<br>DEAE-Dextran 1% | 1 day              | 108  |
|  | 7 days             | 120  |
|  | 14 days            | 114  |
|  | 21 days            | 106  |
|  | 5 months           | 102  |

Unstabilised Enzyme retained 26% activity after 7 days

TABLE 2

| Bulk preparation                         | Incubation<br>37°C | % Activity retained relative<br>to freshly dried enzyme |
|--|--------------------|---|
| DEAE-Dextran 1%<br>Lactitol 10%          | 4 days             | 138   |
| Alcohol oxidase<br>7.25 units/10mg solid | 12 days            | 121   |

Unstabilised Enzyme retained 34% activity after 4 days.

TABLE 3

| Preparation     | Incubation<br>37°C | % Activity remaining relative<br>to freshly dried enzyme |
|-----------------|--------------------|--|
| Choline oxidase | 1 day              | 99   |
| DEAE-Dextran 1% | 5 days             | 84   |
| Lactitol 10%    | 10 days            | 81   |
|                 | 15 days            | 83   |

Unstabilised Enzyme retained 24% of activity after 1 day, decreasing to 11% after 5 days.

TABLE 4

| Preparation                     | Incubation<br>37°C | % Activity remaining relative<br>to freshly dried enzyme |
|---------------------------------|--------------------|--|
| Glycerol 3 phosphate<br>oxidase | 1 days             | 104  |
|                                 | 5 days             | 120  |
| DEAE-Dextran 1%                 | 10 days            | 117  |
| Lactitol 10%                    | 15 days            | 113  |

Unstabilised enzyme retained 94% activity after 1 day but only retained 54% activity after 15 days.

TABLE 5

| Preparation            | Incubation<br>37°C | % Activity remaining relative<br>to freshly dried enzyme |
|------------------------|--------------------|--|
| Alcohol oxidase        | 1 day              | 137  |
| DEAE-Dextran 1% wt/vol | 6 days             | 103  |
| Lactose 10% wt/vol     | 10 days            | 108  |
|                        | 15 days            | 92   |

Unstabilised enzyme retained 23% of activity after 10 days at 37°C.

TABLE 6

| Preparation                            | % Activity remaining (relative to freshly dried enzyme) |                |                 |                   |
|--|---|----------------|-----------------|-------------------|
|  | 1 day<br>37°C   | 6 days<br>37°C | 18 days<br>37°C | 11 months<br>37°C |
| Alcohol oxidase +<br>Dextran 1% wt/vol |   |                |                 |                   |
| M Wt.                                  |   |                |                 |                   |
| T10 10 000                             | 93  | 87             | 73              | 77                |
| T40 40 000                             | 82  | 75             | 61              | 64                |
| T70 70 000                             | 84  | 86             | 65              | 60                |
| T500 500 000                           | 83  | 86             | 62              | 49                |
| T2000 2 000 000                        | 45  | 43             | 22              | 14                |

Unstabilised enzyme retained 30% of activity after 6 days.

TABLE 7Galactose oxidase

Dextran concentrations 5% (M.W 10 000).

Incubation 37°C

% Activity remaining relative  
to activity of freshly dried enzyme.

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|         |     |
|---------|-----|
| 1 day   | 92% |
| 7 days  | 87% |
| 10 days | 82% |

---

TABLE 8

| Preparation                      | Incubation<br>37°C | % Activity remaining relative<br>to freshly dried enzyme |
|----------------------------------|--------------------|--|
| Alcohol oxidase +<br>5% Inositol | 1 Day              | 150%   |
|                                  | 7 days             | 196%   |
|                                  | 14 days            | 166%   |
|                                  | 23 days            | 178%   |

Unstabilised enzyme retained 26% activity after 7 days.

TABLE 9

| Preparation                        | Incubation<br>37°C | % Activity remaining relative<br>to freshly dried enzyme |
|------------------------------------|--------------------|--|
| Galactose oxidase +<br>5% Inositol | 1 day              | 85%  |
|                                    | 4 days             | 78%  |
|                                    | 10 days            | 74%  |

## CLAIMS

1. A method of protecting proteins against denaturation on drying comprising the steps of:  
mixing an aqueous solution of the protein with a soluble cationic polyelectrolyte and a cyclic polyol, and  
removing water from the solution.
2. A method as claimed in Claim 1, wherein said polyelectrolyte comprises a quaternary ammonium functionalised polysaccharide.
3. A method as claimed in Claim 2, wherein said polyelectrolyte comprises diethylamminoethyl-dextran or chitosan.
4. A method as claimed in Claim 1, wherein the polyelectrolyte comprises polyethyleneimine.
5. A method as claimed in any preceding claim, wherein the polyol is a di or trisaccharide.
6. A method as claimed in Claim 5 wherein the polyol is selected from the group comprising: lactitol, lactose, maltose sucrose and cellobiose.
7. A method as claimed in any preceding claim, wherein water is removed at a temperature between 4° and 50°C.
8. A method as claimed in Claim 7, wherein the temperature is 25° to 35°C.
9. A dried product containing a protein, cyclic polyol and a cationic polyelectrolyte.
10. A dried product as claimed in Claim 9 containing an enzyme, cyclic polyol and quaternary ammonium functionalised polymeric polysaccharide.
11. A dried product as claimed in Claim 10, containing an enzyme, cyclic polyol and a polyethyleneimine.
12. A dried product as claimed in any of Claims 9 to 11, wherein the polyol is a di or trisaccharide.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01346

| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup><br>According to International Patent Classification (IPC) or to both National Classification and IPC<br>IPC <sup>5</sup> : C 12 N 9/96  |   |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
|---|---|-------------------------------------|---|--|-------------------------------------|----------|---|---------------------------------|------------------------|---|---|---|---|---|-------|--|--|
| <b>II. FIELDS SEARCHED</b><br><div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System <sup>1</sup></th> <th style="width: 70%; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">IPC<sup>5</sup></td> <td style="border: 1px solid black; padding: 5px;">C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation<br/>to the extent that such Documents are included in the Fields Searched <sup>8</sup></div>  |   |                                     | Classification System <sup>1</sup>                        | Classification Symbols   | IPC <sup>5</sup>                    | C 12 N   |   |                                 |                        |   |   |   |   |   |       |  |  |
| Classification System <sup>1</sup>  | Classification Symbols  |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| IPC <sup>5</sup>  | C 12 N  |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>1</sup></b><br><table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>9</sup></th> <th style="width: 60%; border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 30%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="border: 1px solid black; padding: 5px;">Chemical Abstracts, vol. 89, no. 16,<br/>16 October 1978, (Columbus, Ohio, US),<br/>see page 404, abstract 135880v<br/>&amp; SU, A, 615933 (LENINGRAD<br/>SCIENTIFIC-RESEARCH INSTITUTE OF<br/>HEMATOLOGY AND BLOOD TRANSFUSION)<br/>25 July 1978</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="border: 1px solid black; padding: 5px;">FR, A, 2209776 (AJINOMOTO) 5 July 1974,<br/>see the whole document</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="border: 1px solid black; padding: 5px;">EP, A, 0136996 (MONSANTO CO.)<br/>10 April 1985,<br/>see the whole document</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">4</td> </tr> <tr> <td colspan="3" style="border: 1px solid black; text-align: center; padding: 10px;">-----</td> </tr> </table> |   |                                     | Category <sup>9</sup>                                     | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup> | Relevant to Claim No. <sup>13</sup> | A        | Chemical Abstracts, vol. 89, no. 16,<br>16 October 1978, (Columbus, Ohio, US),<br>see page 404, abstract 135880v<br>& SU, A, 615933 (LENINGRAD<br>SCIENTIFIC-RESEARCH INSTITUTE OF<br>HEMATOLOGY AND BLOOD TRANSFUSION)<br>25 July 1978 | 1                               | A                      | FR, A, 2209776 (AJINOMOTO) 5 July 1974,<br>see the whole document | 1 | A | EP, A, 0136996 (MONSANTO CO.)<br>10 April 1985,<br>see the whole document | 4 | ----- |  |  |
| Category <sup>9</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>  | Relevant to Claim No. <sup>13</sup> |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| A   | Chemical Abstracts, vol. 89, no. 16,<br>16 October 1978, (Columbus, Ohio, US),<br>see page 404, abstract 135880v<br>& SU, A, 615933 (LENINGRAD<br>SCIENTIFIC-RESEARCH INSTITUTE OF<br>HEMATOLOGY AND BLOOD TRANSFUSION)<br>25 July 1978 | 1                                   |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| A   | FR, A, 2209776 (AJINOMOTO) 5 July 1974,<br>see the whole document   | 1                                   |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| A   | EP, A, 0136996 (MONSANTO CO.)<br>10 April 1985,<br>see the whole document   | 4                                   |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| -----   |   |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>14</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>   |   |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| <b>IV. CERTIFICATION</b><br><table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; padding: 5px;">26th February 1990</td> <td style="border: 1px solid black; text-align: center; padding: 5px;">29.03.90</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="border: 1px solid black; text-align: center; padding: 5px;">            T.K. WILDIS         </td> </tr> </table>  |   |                                     | Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report  | 26th February 1990                  | 29.03.90 | International Searching Authority   | Signature of Authorized Officer | EUROPEAN PATENT OFFICE | T.K. WILDIS   |   |   |   |   |       |  |  |
| Date of the Actual Completion of the International Search   | Date of Mailing of this International Search Report   |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| 26th February 1990  | 29.03.90  |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| International Searching Authority   | Signature of Authorized Officer   |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |
| EUROPEAN PATENT OFFICE  | T.K. WILDIS   |                                     |   |  |                                     |          |   |                                 |                        |   |   |   |   |   |       |  |  |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 8901346

SA 32768

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/03/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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|   |                     | DE-A- 2361170              | 27-06-74            |
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|   |                     | JP-A- 60156397             | 16-08-85            |